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In Vitro *Plasmodium falciparum* Drug Sensitivity Assay: Inhibition of Parasite Growth by Incorporation of Stomatocytogenic Amphiphiles into the Erythrocyte Membrane

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Lupeol, which shows in vitro inhibitory activity against *Plasmodium falciparum* 3D7 strain with a 50% inhibitory concentration (IC₅₀) of 27.7 ± 0.5 μM, was shown to cause a transformation of the human erythrocyte shape toward that of stomatocytes. Good correlation between the IC₅₀ value and the membrane curvature changes caused by lupeol was observed. Preincubation of erythrocytes with lupeol, followed by extensive washing, made the cells unsuitable for parasite growth, suggesting that the compound incorporates into erythrocyte membrane irreversibly. On the other hand, lupeol-treated parasite culture continued to grow well in untreated erythrocytes. Thus, the antiparasitic activity of lupeol appears to be indirect, being due to stomatocytic transformation of the host cell membrane and not to toxic effects via action on a drug target within the parasite. A number of amphiphiles that cause stomatocyte formation, but not those causing echinocyte formation, were shown to inhibit growth of the parasites, apparently via a mechanism similar to that of lupeol. Since antiparasitic agents that inhibit parasite growth through erythrocyte membrane modifications must be regarded as unsuitable as leads for development of new antimalarial drugs, care must be exercised in the interpretation of results of screening of plant extracts and natural product libraries by an in vitro *Plasmodium* toxicity assay.

Malaria continues to be a growing health problem of global dimensions. According to World Health Organization estimations, the number of clinical cases has reached 300 to 500 million per year, most of which are in sub-Saharan Africa. Increased geographical spreading of the disease is observed, mainly in Asia. In addition to a mortality rate of 1.1 to 2.7 million deaths per year (27), mostly among children, malaria puts a heavy economic burden on the developing world by exhausting health system resources and by associated loss of economic activity. Only a limited number of chemotherapeutic agents for the treatment of malaria are available, and the growing problem of drug resistance makes adequate treatment of malaria increasingly difficult (27).

In the absence of a functional, safe, and widely available malaria vaccine, efforts to develop new antimalarial drugs are profoundly important. Since the vast majority of the existing antimalarial chemotherapeutic agents are based on natural products (3, 4), biological chemodiversity continues to play an important role in the search for leads for antimalarial drugs.

Research aiming at drug discovery and the development of novel antimalarial agents depends critically on in vitro toxicity assays employing various *Plasmodium* strains, pioneered by Desjardins et al. (6). As with any cellular in vitro assay, the *Plasmodium* assay should be fast, robust, and not prone to artifacts. Ideally, an assay should be able to identify drugs that specifically interfere with the parasite biochemistry. However, since the *Plas-*

modium parasites are cultured in erythrocytes, their growth may be influenced indirectly by drug effects on the host cell. Because *Plasmodium* parasites depend on the function of the erythrocyte membrane by changing its permeability and opening nutrient uptake channels (5), alterations of membrane properties are likely to interfere with the parasite growth. This may happen generally with surface-active (amphiphilic) compounds and other lipophilic compounds that can be incorporated into the lipid bilayer. In this work we describe a correlation between changes of the erythrocyte membrane shape observed microscopically and the inhibition of *Plasmodium falciparum* growth caused by lupeol (Fig. 1), a natural product isolated as the principal in vitro antiparasitic agent from the extract of a tropical plant *Rinorea ilicifolia* Kuntze (plant family *Violaceae*). Similar results were obtained with a series of synthetic amphiphiles. The indirect antiparasitic activity, hardly of any interest in a search for new antimalarial drugs, must be taken into account when the results of in vitro drug sensitivity assays based on *Plasmodium* cultures grown in erythrocytes are assessed.

MATERIALS AND METHODS

Chemicals. Authentic lupeol, *N*-cetyl-*N,N,N*-trimethylammonium bromide (compound 5), chlorpromazine hydrochloride (compound 11), and chloroquine diphosphate were purchased from Sigma Chemical Co. Zwittergents [3-(alkyldimethylammonio)-1-propanesulfonates (compounds 3 and 4)] and decyl-β-D-glucopyranoside (compound 7) were obtained from Calbiochem-Behring, and sodium alkyl sulfates (compounds 1 and 2) were obtained from Merck. Hexadecylpyridinium bromide (compound 6), octaethyleneglycol monoalkyl ethers (compounds 8 and 9), and pentaethyleneglycol monododecyl ether (compound 10) were from Fluka. All chemicals were reagent grade and were used as received. Column-chromatographic separations were performed on Matrex silica gel 60A (37 to 70 μm; Millipore). [2,3,4,5,6-³H]phenylalanine was obtained from Amersham Pharmacia Biotech, and the solution was diluted to 1.6 MBq/ml with

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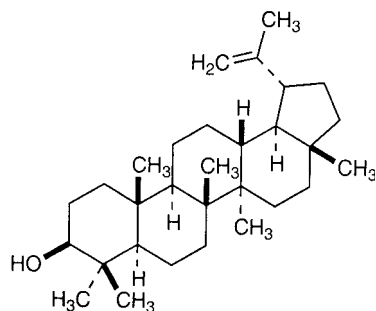


FIG. 1. Chemical structure of lupeol.

saline. Growth media and auxiliary chemicals for parasite cultivation were purchased from Gibco-BRL.

Assay for antiparasmodial activity. A modification of Desjardins' radioisotope method (6) for measuring the growth of a chloroquine-sensitive strain of *P. falciparum* (3D7) was adopted, with incorporation of [^3H]phenylalanine as an index of growth (11). Thus, 50 μl of the growth medium (RPMI 1640 with HEPES and supplemented with 0.45% Albumax II, 1.42 mM L-glutamine, 133 μM hypoxanthine, and 44 mg of gentamicin sulfate/ml) containing test substances added from a dimethyl sulfoxide (DMSO) stock (lupeol) or a water stock (amphiphiles) was mixed with 50 μl of a suspension of parasitized erythrocytes (blood type, O; hematocrit, 5%; parasitemia, 2 to 3%) in 96-well microtiter plates. The maximum final concentration of DMSO in the growth medium was 0.5%; reference wells contained DMSO in the concentration range of 0 to 0.5%. Each concentration of the test substances was tested in duplicate. The plates were incubated at 37°C for 24 h in an atmosphere consisting of 93% N_2 , 5% CO_2 , and 2% O_2 ; [^3H]phenylalanine solution (25 μl , 1.6 MBq/ml) was added, and the incubation was continued for 24 h. The cells were harvested on filter plates (Packard UniFilter GF/C) with Packard Cell Harvester (FilterMate 96-well model) by using distilled water as a wash medium, and 20 μl of scintillation fluid (Microscint-O) was added to each filter well. The incorporation of activity was measured by using a TopCount Packard Microplate Scintillation Counter. The 50% inhibitory concentration (IC_{50}) values were derived from the radioactivity counts by nonlinear curve fitting with GraFit program (version 4.06) from Erithacus Software, Ltd., by using a four-parameter logistic equation. Chloroquine was used as a reference in all determinations. Each assay was repeated at least twice ($n \geq 2$).

Erythrocyte membrane shape changes caused by lupeol. Normal and parasitized erythrocytes were incubated in medium containing various amounts of lupeol (3.13 to 200 $\mu\text{g/ml}$) or DMSO in the concentration range from 0 to 2% as described above, but omitting addition of [^3H]phenylalanine. After 48 h of incubation, the plate was placed on a shaking table for 1 min, and 20- μl samples were spread on microscope slides, allowed to dry, fixed with methanol, and stained with Giemsa for phase-contrast light microscopy. For transmission electron microscopy, 100- μl samples were transferred into Eppendorf tubes and treated with 5 μl of 25% aqueous glutaric aldehyde for at least 1 h. A fraction (5 μl) of this suspension was used for light-microscopic determination of the erythrocyte shape in a hanging drop. The cells were washed three times with 0.1 M sodium phosphate buffer (pH 7.4). After postfixation with osmium tetroxide (a 1:1 mixture of 2% aqueous OsO_4 and 0.3 M sodium phosphate buffer [pH 7.4]) for 1 to 2 h and repeated washing with phosphate buffer, the cells were dehydrated by a series of acetone washes and embedded in Epon. The samples were cut in 50- to 60-nm-thick sections, stained with lead citrate and then with uranyl acetate, and examined with a JEOL 100SX electron microscope.

Parasite growth in erythrocytes pretreated with lupeol. Solutions of lupeol in 0.4 ml of DMSO were mixed with 38.6 ml of RPMI 1640 medium (containing all additives) and 1 ml of packed human erythrocytes (10^{10} cells), and the suspensions (final hematocrit of 2.5%) were placed in 80- cm^2 flasks and incubated for 48 h at 37°C (93% N_2 , 5% CO_2 , and 2% O_2). The final concentration of lupeol was 25 or 100 $\mu\text{g/ml}$; reference flasks contained the same amount of DMSO but no lupeol. The erythrocytes were separated (2,000 rpm for 10 min) and washed four times with the medium in order to remove dissolved lupeol. The process was monitored microscopically to ensure that no cell shape alterations occurred during this procedure. The cells were then used for subcultivation of *P. falciparum* in 25- cm^2 flasks. Thus, 200 μl of the erythrocytes obtained as described above was mixed with 20 μl of infected erythrocytes (parasitemia 5%) and incubated with 5 ml of the RPMI medium. The growth medium was replaced with a fresh portion every 48 h. Every 2 to 3 days a sample of erythrocytes was withdrawn, and parasite counts were deter-

mined microscopically, counting three microscope fields ($n = 3$, 100 erythrocytes per field). When parasitemia reached 5%, the culture was subcultured similarly as described above by using 5 μl of the culture.

Growth of lupeol-treated parasites in untreated erythrocytes. Solutions of lupeol in 50 μl of DMSO were mixed with 5 ml of RPMI 1640 medium (containing all additives) and 100 μl of parasitized erythrocytes (parasitemia 5.4%), and the suspensions (final hematocrit 2.5%) were placed in 25- cm^2 flasks and incubated for 3 or 6 h as described above. The final concentration of lupeol was 25 or 100 $\mu\text{g/ml}$; reference flasks contained the same amount of DMSO but no lupeol. The erythrocytes were separated and washed as described previously in order to remove all traces of lupeol. The process was evaluated microscopically to monitor the cell shape. The parasitized cells thus obtained were subcultured with untreated erythrocytes. The growth medium was replaced with a fresh portion every 48 h. A sample of erythrocytes was withdrawn every day, and parasite counts were determined microscopically, counting three microscope fields ($n = 3$, 100 erythrocytes per field), until parasitemia reached 5%.

Isolation of lupeol from *R. ilicifolia* Kuntze. Plant material was collected in Ghana and was identified by D. K. Abbiw, Department of Botany, University of Ghana. Voucher specimen (GC47600) was deposited in the Ghana Herbarium (Department of Botany, University of Ghana, Legon, Ghana). Finely ground branches (258 g) were degreased for 2 h in a Soxhlet apparatus with 1.5 liter of light petroleum (boiling point, 40 to 65°C) and then extracted three times by overnight soaking in 1 liter of 96% ethanol. The ethanol extracts were combined and evaporated to dryness, and the residue (9.6 g) was distributed between ethyl acetate and water. Evaporation gave 2.2 g of the residue from the ethyl acetate fraction and 6.0 g from the aqueous fraction. Antiplasmodial activity was associated solely with the ethyl acetate fraction ($\text{IC}_{50} < 12 \mu\text{g/ml}$). This material was chromatographed on a 2-by-72-cm silica gel column (170 g of silica) by using a step gradient of ethyl acetate in dichloromethane, collecting 25-ml fractions which were monitored by thin-layer chromatography and by *P. falciparum* growth inhibition assay. The most active fractions, eluted with 3% ethyl acetate, yielded 62 mg (0.024% of the plant material used) of lupeol, as identified by comparison of its 400-MHz ^1H and 100.6-MHz ^{13}C nuclear magnetic resonance spectra in CDCl_3 with those of authentic lupeol.

RESULTS

Lupeol as apparent antiparasmodial constituent of *R. ilicifolia*. In a screening of plants for antiparasmodial activity, extracts of *R. ilicifolia* completely inhibited the incorporation of

TABLE 1. Apparent in vitro inhibitory activity of lupeol and model amphiphiles on the growth of *P. falciparum* 3D7 and the effect of the compounds on human erythrocyte shape

Compound ^a	Net charge	Mean IC_{50} (μM) \pm spread ($n = 2$)	Effect on erythrocyte shape ^b
Lupeol	0	27.7 \pm 0.5	Stomatocytogenic
1	-1	460 \pm 17	Echinocytogenic
2	-1	$\gg 145^c$	Echinocytogenic
3	0	782 \pm 140	Echinocytogenic
4	0	$\gg 65^d$	Echinocytogenic
5	+1	20.5 \pm 1.9	Stomatocytogenic
6	+1	1.4 \pm 0.17	Stomatocytogenic
7	0	233 \pm 13	Stomatocytogenic ^f
8	0	54.6 \pm 7.8	Stomatocytogenic
9	0	33.1 \pm 2.6	Stomatocytogenic
10	0	67.7 \pm 2.7	Stomatocytogenic
11	+1	27.9 \pm 2.6	Stomatocytogenic
Chloroquine	+2	0.045 \pm 0.004	None ^e

^a Compounds 1 to 11 correspond to the numbered structures in Fig. 4.

^b Data for compounds 1 to 11 are from references 13 and 18.

^c There was practically no inhibition at this concentration, which is the solubility limit of the compound in the medium.

^d There was practically no inhibition at this concentration; lysis of erythrocytes occurs at higher concentrations.

^e No effect on membrane shape at active concentrations ($\leq 2 \mu\text{M}$) was observed (this study); the compound is stomatocytogenic at millimolar concentrations (22).

^f Weakly.

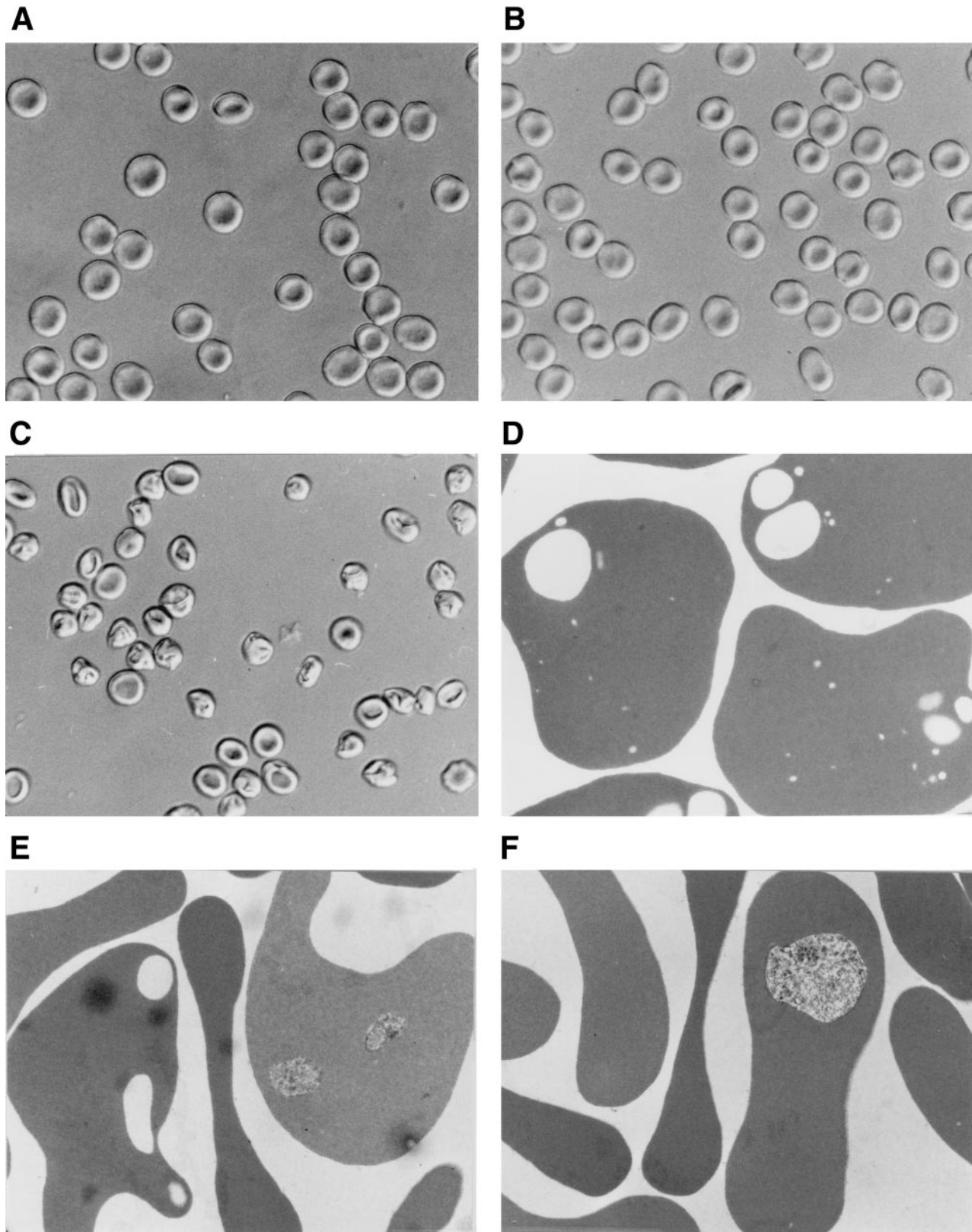


FIG. 2. Effect of lupeol on human erythrocyte shape. (A) Control. (B) Erythrocytes treated with 2% DMSO. (C and D) Erythrocytes treated with 50 μg of lupeol/ml (117.2 μM). (E) Erythrocytes treated with 12.5 μg of lupeol/ml (29.3 μM). (F) Erythrocytes treated with 3.13 μg of lupeol/ml (7.3 μM). Micrographs A to C were obtained with a light microscope ($\times 800$), with a sample fixed with glutaric aldehyde (final concentration, 1%) and sealed between glass slides in order to prevent the sample from drying. Micrographs D to F were obtained with a transmission electron microscope ($\times 10,000$).

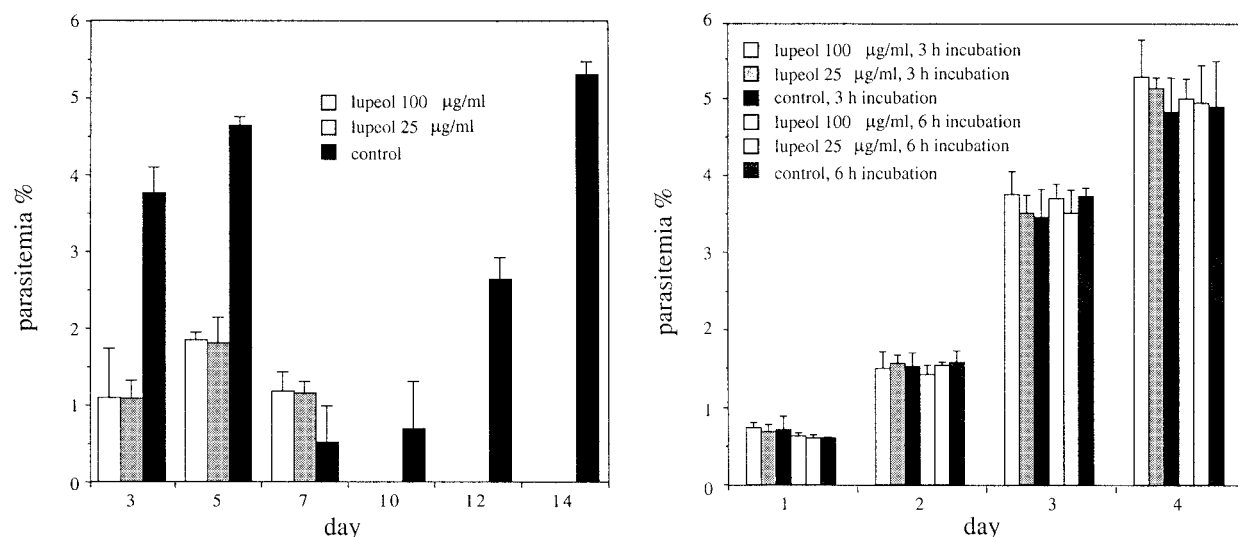


FIG. 3. (Top panel) Growth of *P. falciparum* 3D7 in erythrocytes preincubated with 100 µg (234.4 µM) and 25 µg (58.6 µM) of lupeol/ml and in erythrocytes preincubated in an identical way but in the absence of lupeol (control). The control was subcultured on day 5. (Bottom panel) Growth in untreated erythrocytes of *P. falciparum* 3D7 preincubated with lupeol. The parasite cultures were pretreated with 25 µg (58.6 µM) or 100 µg (234.4 µM) of lupeol/ml for 3 or 6 h and then subcultured with fresh erythrocytes.

[³H]phenylalanine into the chloroquine-sensitive 3D7 strain of *P. falciparum* at 12 µg/ml. Bioactivity-guided fractionation identified the triterpene lupeol (Fig. 1) as the major agent responsible for the activity, with an apparent IC₅₀ value of 11.8 µg/ml or 27.7 µM (Table 1) obtained with purified material. After lupeol was identified as the antiplasmodial agent of *R. ilicifolia* extracts, all subsequent experiments were carried out with commercial samples of lupeol.

Effect of lupeol on erythrocyte membrane. Microscopic examination of a parasite culture incubated with lupeol showed concentration-dependent changes of erythrocyte shape. No lysis of the cells was observed, but the shape of erythrocytes was altered toward stomatocytic forms. The changes were very pronounced at lupeol concentrations of ≥50 µg/ml (117.2 µM) (Fig. 2) but could also be observed at concentrations as low as 3.13 µg/ml (7.3 µM). Consistent findings were obtained by various methods of sample fixation, and thus the changes in erythrocyte shape were not induced by sample preparation. Progressive changes in erythrocyte shape were observed by transmission electron microscopy to be a function of concentration (Fig. 2). Based on the nomenclature of Bessis (2), the dominant erythrocyte shapes were the normal discocytes at lupeol concentrations of 3.13 µg/ml (7.3 µM), which changed to spherostomatocytes at 12.5 to 200 µg/ml (29.3 to 468.7 µM). At the lowest lupeol concentration (3.13 µg/ml), only a few stomatocytes of type 1 (S1) were observed. At 6.25 µg of lupeol/ml (14.6 µM) discocytes were still present, but stomatocytes of type 2 (S2) predominated. At 12.5 µg/ml, a few S1 were seen, but spherostomatocytes (SS) were the dominant shape. Only SS forms were seen at higher concentrations (25 to 200 µg/ml, 58.6 to 468.7 µM). Thus, the changes of erythrocyte membrane curvature occur at concentrations corresponding to and below the apparent IC₅₀ value of lupeol. As a result of stomatocytosis lupeol induced endovesiculation, and the number as well as the size of the vesicles increased with increasing lupeol concentration (Fig. 2). The vesicles were located mainly

along the erythrocyte membrane. Parasites observed inside the lupeol-treated erythrocytes were fewer in number and much smaller than those observed inside untreated erythrocytes. DMSO at concentrations up to 2% caused no changes in erythrocyte shape (Fig. 2). The effect of lupeol on erythrocyte shape did not depend on whether the cells contained the parasites or not. No effect on erythrocyte shape was observed with chloroquine in the concentration range that caused complete inhibition of parasite growth.

Effect of pretreatment of erythrocytes with lupeol on the growth of *P. falciparum*. When parasites were cultured in erythrocytes pretreated with lupeol and extensively washed prior to cultivation, severe impairment of growth was observed (Fig. 3). Thus, when parasitized erythrocytes (parasitemia, 5%) were mixed with erythrocytes exposed to 25 µg of lupeol/ml (58.6 µM), all forms of the parasites were observed only during the first 3 days of cultivation. In the culture with erythrocytes pretreated with 100 µg of lupeol/ml (234.4 µM), mainly trophozoites were observed during the first days. There appears to be a slight increase of parasitemia from day 3 to day 5 in both cultures. After an additional 2 days, most of the parasites were extracellular, and no live parasites were observed from day 10 in either culture (the medium was replaced regularly). Culture with erythrocytes pretreated with 100 µg of lupeol/ml contained more extracellular parasites than that pretreated with 25 µg/ml, and both cultures showed an excess of ruptured schizonts and merozoites compared to the control. On the other hand, parasites cultured with erythrocytes pretreated in the identical way but without lupeol present exhibited normal growth, reaching 5% parasitemia on day 5, after which they were subcultured with the same batch of erythrocytes and continued to grow normally. This experiment demonstrates that exposure to lupeol caused permanent changes of erythrocytes that made them unsuitable as parasite host cells even in the absence of lupeol in the growth medium. Normal growth of parasites in the cells pretreated the same way but in the ab-

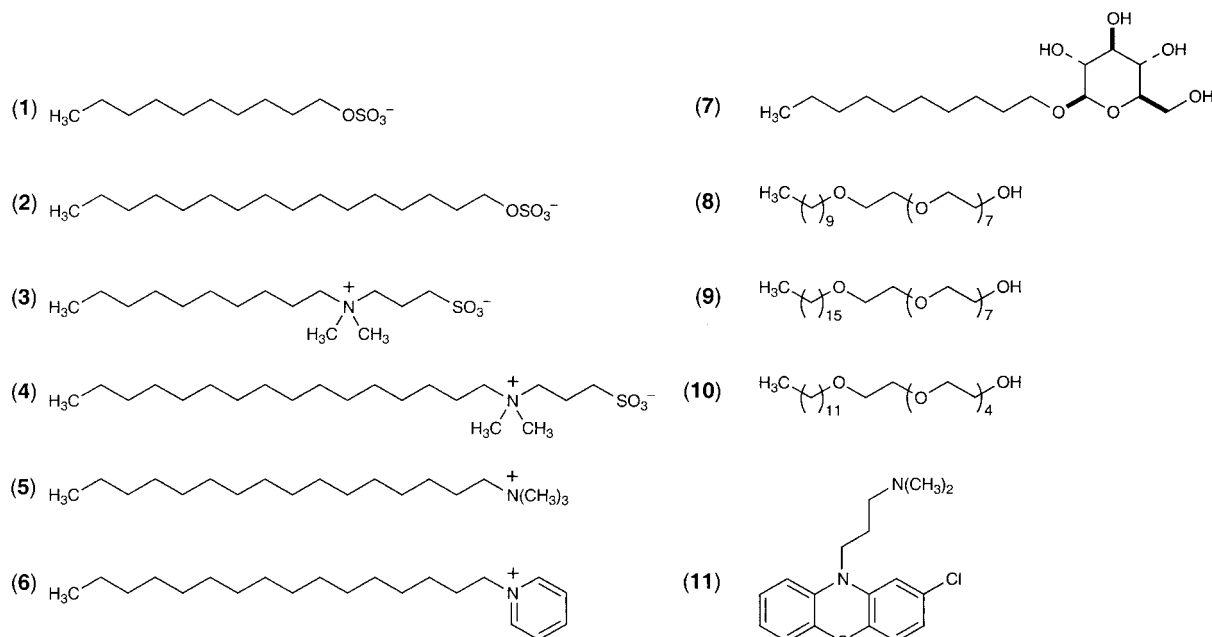


FIG. 4. Structures of amphiphilic compounds 1 to 11 tested for antiplasmodial activity in vitro.

sence of lupeol ensured that erythrocytes were not damaged during the washing procedure. The latter conclusion was supported by the microscopic examination of the cell shapes.

Effect of pretreatment with lupeol on parasite growth in untreated erythrocytes. When erythrocytes were incubated for 3 or 6 h with 25 or 100 μg of lupeol/ml (58.6 or 234.4 μM), the culture showed stomatocytic cell shape, whereas normal discocytic shapes were observed with the control cells. When the cultures were subcultured with fresh, untreated erythrocytes, all cultures continued to grow at the same rate, regardless of whether they were pretreated with lupeol or not (Fig. 3). All of the cultures contained all parasite stages. Thus, a sublethal preincubation of parasites with lupeol did not affect their ability to invade and multiply in untreated erythrocytes.

Effect of amphiphiles on parasite growth. A number of synthetic amphiphiles (structures 1 to 10 in Fig. 4) known to induce changes in erythrocyte shape (18) were tested for inhibition of *P. falciparum* growth in the in vitro assay. Chlorpromazine (structure 11 in Fig. 4), a standard stomatocytogenic compound (18), was included in the study as a reference. The results are shown in Table 1. There appears to be a good correlation between the in vitro activity of the amphiphiles and the type of membrane shape change induced, with the stomatocytogenic amphiphiles being far more potent antiplasmodial agents than the echinocytogenic amphiphiles.

DISCUSSION

It is well known that erythrocytes respond to various treatments by changing their shape. Echinocytes are produced by forces that cause evagination, whereas stomatocytes are produced by forces that produce invagination of the cellular membrane (9, 16, 23). A variety of amphiphilic agents have been shown to induce echinocytic or stomatocytic shapes of erythrocytes (7, 10, 18). These shape transformations are thought to

depend mainly on the distribution of the amphiphile in the bilayer, i.e., whether the amphiphile is predominantly incorporated into the outer or the inner membrane leaflet. This leads to expansion of one leaflet relative to the other. In the case of charged amphiphiles, there is a correlation between the charge and the membrane shape change effect, presumably governed by electrostatic interactions with negatively charged phospholipids present mainly in the inner leaflet (15). For neutral amphiphiles, no clear structure-activity relationships have emerged. Moreover, amphiphiles at low (sublytic) concentrations protect erythrocytes against hypotonic hemolysis (17, 19). The incorporation of amphiphiles into the erythrocyte membrane was also observed directly by using radioactive tracers (14). There is thus considerable knowledge regarding the changes in erythrocyte membrane shape caused by amphiphiles and concentration regions at which these changes occur.

Using a series of amphiphiles (Fig. 4) previously shown to alter the shape of erythrocytes, we found that forces causing stomatocytic, but not echinocytic, changes of erythrocyte membrane make the cells unsuitable as *P. falciparum* hosts. Thus, stomatocytogenic compounds were shown to act generally as in vitro antiplasmodial agents with moderate to high activity (IC_{50} values in the range of 1.4 to 68 μM ; Table 1). Echinocytogenic compounds were only active at much higher concentrations ($\text{IC}_{50} \geq 460 \mu\text{M}$; Table 1). Alterations of the erythrocyte shape were also observed with lupeol (Fig. 1), the major antiplasmodial constituent isolated from the plant *R. ilicifolia*. It has previously been described that lupeol exhibits inhibitory activity on *P. falciparum* growth in vitro but lacks in vivo activity in mice infected with *P. berghei* (1). However, no information about the mechanism of the in vitro activity was reported. It is now shown that lupeol causes membrane shape changes of erythrocytes toward stomatocytic forms observable at concentrations below its IC_{50} value. The compound induced endovesiculation, which is characteristic of stomatocytogenic

compounds (13). There is a good correlation between the lupeol concentration at which morphological changes in erythrocytes occur and the observed IC_{50} of this compound (Fig. 1 and Table 1). This strongly suggests that the in vitro antiparasitocidal activity of lupeol is indirect, being due to membrane modification of the host cell.

The structure of lupeol is reminiscent of that of cholesterol, and the compound is expected to be able to enter the cellular membranes. Due to the presence of a single hydroxy group and a large, apolar skeleton (Fig. 1), lupeol acts as an amphiphile. According to the bilayer hypothesis (24, 25), stomatocytes are generally formed when a lipophilic compound is incorporated into and expands the inner layer of the lipid membrane. Such changes appear to be more prohibiting with respect to parasite growth than incorporation of an amphiphile into the outer layer, as in case of echinocytogenic compounds (Table 1).

We demonstrated that the inhibition of parasite growth does not require the presence of lupeol in the growth medium, since erythrocytes preincubated with lupeol proved to be unsuitable for parasite cultivation (Fig. 3). This strongly suggests the permanent incorporation of lupeol into the lipid bilayer. The presence of an excess of extracellular merozoites in a culture employing erythrocytes pretreated with lupeol suggests that the invasion of the erythrocytes has been impaired also.

In an inverse experiment relative to that described above, a parasite culture was treated with lupeol and subcultured with untreated erythrocytes (Fig. 3). In this experiment, the time of preincubation had to be limited to 3 to 6 h; otherwise, the parasites would die. In spite of the pretreatment with lupeol, the parasites grew normally in untreated cells after removal of lupeol (Fig. 3). Thus, the ability of the parasites to invade and grow in fresh erythrocytes was not impaired by the initial exposure to lupeol.

Previous studies have demonstrated that alterations of the erythrocyte membrane such as cross-linking of spectrin, changes in deformability, spherocytosis, and modification of the cytoskeletal proteins have inhibitory effects on invasion (8, 11, 20, 21). To our knowledge, no studies of incorporation of lupeol into erythrocytes and its effect on parasite proliferation have been reported prior to this work. A recent report of Vidaya et al. (26) showing that erythrocytes of rats fed with lupeol exhibit altered osmotic fragility is compatible with our findings.

Although the exact mechanism by which stomatocytosis makes the erythrocytes unfavorable for *P. falciparum* invasion and growth has yet to be elucidated, the present findings are of interest for drug discovery programs based on natural products. Lupeol and other pentacyclic triterpenes and sterols with related structures are very common constituents of plants and are thus frequently encountered in plant extracts used for screening. Many synthetic drug candidates may also act as stomatocytogenic amphiphiles. The membrane alterations that inhibit parasite growth take place long before they can be detected by routine examination by optical microscopy (Fig. 2), and thus care has to be exercised when *P. falciparum* in vitro growth inhibition results are interpreted.

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